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DESCRIPTION

MULTIPLE GENE TRANSCRIPTION ACTIVITY ASSAY SYSTEM

TECHNICAL FIELD

The present invention relates to a gene construct for multiply detecting gene transcription activities in living cells by the use of luciferases which emit different color lights, an expression vector containing the construct, transformed mammalian cells containing the construct or the expression vector, a screening method of drugs using the mammalian cells, and a system for multiply determining the transcription activities of respective promoters.

The invention also relates to a gene and a polypeptide used for a system where the transcription activity in the living cells is detected by the use of the luciferase which emits red, orange or blue color light.

BACKGROUND ART

In the life science field, a transcription activity of an intracellular gene has been generally determined, and used for evaluation of exogenous factors given to cells, and analyses of intracellular signal transduction or expression of an individual protein group. The gene transcription activity has been directly determined by Western blotting and the like, or indirectly determined using a luciferase gene or a light-emitting enzyme gene as a reporter gene. In particular, it has been generalized to quantify the transcription activity based on an emitted light intensity using a firefly light-emitting enzyme gene. A fluorescent protein exhibits a fluorescent activity without need of a cofactor almost simultaneously with its intracellular expression. The fluorescent protein has been used as a monitor protein for examining a localization of a protein by the use of the fluorescent activity in the cell as an indicator, but it is difficult to quantify it, and it is unlikely to use it as the reporter gene for the gene expression.

It is important to analyze a quantitative and temporal dynamic change of the protein gene expression, but the transcription activity one gene has been primarily analyzed in conventional reporter techniques. However recently, a system (dual assay system, Promega) for determining two transcription activities by introducing two gene constructs into the cell, i.e., A transcription active region being inserted in a firefly light-emitting enzyme gene and B transcription active region being inserted in a *Renilla* light-emitting enzyme gene has been commercially available. However, this method is a system for determining the transcription activity by adding different luminescent substrates, respectively, two activities can not be determined simultaneously, and only two transcription activities can be determined. Furthermore, since a firefly luciferase is used, a wavelength thereof is changed due to pH and accurate determination is difficult.

Multiple signals are trafficked in a cell, and it is essential to construct a technique to quantitatively determine the multiple transcription activities. For example, in a human biological clock, a Per gene which gives a 24 hour rhythm is controlled by Clock and BMAL gene products. Thus, to precisely evaluate the biological clock, it is essential to determine multiple, at least three transcription activities. Until now, the transcription activity of an individual gene has been determined by the use of a firefly luciferase reporter gene, but a dynamic of only one gene transcription has been observed at a time, and an interaction of biological clock-related gene expressions has remained unclear.

Canceration progresses by abnormal growth of cells caused with activation of an oncogene or by the abnormal growth of the cells due to the control release caused along with inactivation of a tumor suppressing gene. Thus, to evaluate canceration factors and intracellular signal transduction of the canceration, it is desirable to determine the gene transcription activity of the oncogene, the tumor suppressing gene and a mitotic marker

gene. However, in the conventional method, the dynamic of only one gene transcription has been observed at a time, the transcription activities of the three gene can not be evaluated at a time, and thus the interaction of the three genes involved in the canceration has not been sufficiently understood.

The transcription of a gene is caused by binding a substance which suppresses or promotes the gene expression to a particular sequence present on a gene sequence referred to as a promoter region upstream of a gene product. An E-box and a cAMP-binding site are representatives thereof. The gene transcription activity is determined by inserting a certain length of the promoter region into an upstream of a reporter gene. Furthermore, a particular sequence believed to be effective is then synthesized, and inserted into the upstream of the reporter gene to examine an effect of the particular sequence. To examine a transcription controlling effect of the particular sequence, it is necessary to simultaneously evaluate the transcription activity of the original promoter region and the transcription activity capable of standardizing the effect in combination. However, in the conventional method, the transcription dynamic of only one gene has been observed, and the particular sequence for the control of the transcription activity can not be sufficiently evaluated.

A luciferase is useful as a means to directly observe the gene transcription activity in the cells, and has been used as a detection monitor protein of the gene expression. There are a wide variety of luciferases, but no reporter gene for determining the transcription activity based on their diversity is available. If using luciferase genes which emit different color lights as the reporter genes and different transcriptional active regions are inserted into mammalian cells, then multiple transcription activities can be determined. A red-emitting luciferase derived from a rail road worm has the longest wavelength of luminescence, is easily discriminated compared to the luciferases derived from a firefly and a click beetle, and is highly permeable into the

cell due to the red-emitting color. However, the expression of the red and green-emitting luciferases from the rail road worm has been successfully done only in *Escherichia coli* (US 2002/0119542-A1), and there is no successful example as the system in the mammalian cells including human cells.

There is also an example in which the expression of a luciferase gene from the rail road worm in the mammalian cells was enabled by modifying a structure of the gene (WO 2003/016839).

As the luciferase, a luciferase derived from *Rhagophthalmus ohba* has been also known.

The expression of a green-emitting luciferase derived from *Rhagophthalmus ohba* has been successfully done in only *Escherichia coli* (Ohmiya, Y. Sumiya, M. Viviani, VR. and Ohba N.; Comparative aspects of a luciferase molecule from the Japanese luminous beetle *Rhagophthalmus ohba*. Sci. Rept. Yokosuka City Mus..47, 31-38, 2000). Based on this sequence, an orange-emitting luciferase derived from *Rhagophthalmus ohba* was created and the expression thereof was also successfully done in *Escherichia coli* (Viviani, VR., Uchida, A., Suenaga, N., Ryufuku M. and Ohmiya Y.: Thr-226 is a key-residue for bioluminescence spectra determination in beetle luciferases. Biochem. Biophys. Res. Commun., 280, 1286-1291, 2001). Additionally, as the luciferase, blue-emitting luciferases derived from a dinoflagellate and *Renilla* have been also known.

It is an object of the present invention to construct and optimize a reporter gene capable of determining or quantifying multiple transcription activities in a cell simultaneously or at the same phase, further develop a multiple gene transcription activity determining system using the reporter gene group, and utilize the same for cell functional analyses in life science, further the treatment/examination of pathology and new drug development.

It is also another object to make a gene construct by which a red- or a green-emitting luciferase from a rail road worm is stably transcribed and stably translated in mammalian cells or in

animals.

It is also another object to make a gene construct by which an orange or a green-emitting luciferase from a *Rhagophthalmus ohba* is stably transcribed and stably translated in mammalian cells or in animals.

This enables to stably determine and visualize a change of the gene transcription activity in the mammalian cells or in the animals.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an outline of determination for multiple gene transcription activities and differences from a conventional method.

Fig.2 shows a structure of an expression vector for mammalian cells and luminescence activity in Hela cells.

Fig. 3 shows luminescence spectra of a red-emitting luciferase and a green-emitting luciferase from a rail road worm produced in cultured mammalian cells.

Fig. 4 shows intracellular lifespan of a red-emitting luciferase and a green-emitting luciferase from a rail road worm produced in cultured mammalian cells.

Fig. 5 shows a simultaneous luminescence spectrum of a red-emitting luciferase and a green-emitting luciferase produced in cultured mammalian cells and property of a filter used for color identification (transmittance of lights).

Fig. 6 shows luminescence reaction curves and luminescence activity determining time of a red-emitting luciferase and a green-emitting luciferase.

Fig. 7 shows an abundance ratio and luminescence activity of a red-emitting luciferase and a green-emitting luciferase (in the case of using the filter in Fig. 5).

Fig. 8 shows a result of an actual multiple transcription activity determination, i.e., simultaneously determining two transcription activities by lights from a red-emitting luciferase and a green-emitting luciferase, determining the transcription

activity of a standard gene by the light from a blue-emitting luciferase, and standardizing the two transcription activities.

Fig. 9 shows a simultaneous luminescence spectrum of a red-emitting luciferase, a green-emitting luciferase and a blue-emitting luciferase produced in cultured mammalian cells and property of filters used for color identification (transmittance of lights).

Fig. 10. shows a result indicating that transcription activities shown by a red-emitting luciferase and a green-emitting luciferase were obtained from continuous monitoring of the two transcription activities.

Fig. 11 shows an example in which many specimens are exhaustively analyzed by a primary screening.

Fig. 12 shows an example in which an individual event is evaluated by a secondary screening.

Fig. 13 shows homology of a DNA sequence of a rail road worm red-emitting luciferase gene mutant (SEQ ID NO:7) of the present invention with that of a rail road worm wild-type red-emitting luciferase gene (SEQ ID NO:3).

Fig. 14 shows homology of a DNA sequence of a rail road worm red-emitting luciferase gene mutant (SEQ ID NO:7) having a maximum luminescence wavelength of 630 nm with that of a rail road worm red-emitting luciferase gene mutant of WO 2003/016839 (SEQ ID NO:6) having a maximum luminescence wavelength of 622 nm.

Fig. 15 shows differences in luminescence activity of a wild-type rail road worm red-emitting luciferase and a mutant rail road worm red-emitting luciferase.

Fig. 16 shows luminescence spectra of a mutant (SEQ ID NO:7) introduced and produced in mammalian cells (mouse NIH3T3 cells, a thick line) and a rail road wild-type (SEQ ID NO:3) produced in insect silk worm cells (a thin line).

Fig. 17 shows homology of a DNA sequence of a *Rhagophthalmus ohba* green-emitting luciferase mutant (SEQ ID NO:10) with that of a wild-type (SEQ ID NO:8).

Fig. 18 shows difference in luminescence activity of

Rhagophthalmus ohba green-emitting luciferase wild-type and mutant and *Rhagophthalmus ohba* orange-emitting luciferase wild type and mutant.

Fig. 19 shows an outline of a method for detecting the expression of three genes by one substrate, a firefly luciferin. In the method of the present embodiment, determination is performed by transmitting three transcription activities by red, orange and green color lights and identifying the respective colors.

Fig. 20 shows luminescence spectra of a mixture of *Rhagophthalmus ohba* green and orange-emitting, rail road worm red-emitting luciferases and a transmittance curve of a set split filter.

Fig. 21 shows abundance ratio and luminescence activity (when using the filter shown in Fig. 22) of two color luciferases (combinations of (A) green-red-emitting, (B) green-orange-emitting and (C) orange-red-emitting luciferases).

Fig. 22 shows an abundance ratio and luminescence activity (when using the filter shown in Fig. 20) of three color luciferases ((A) green-orange-emitting luciferases when a red-emitting luciferase is 1; (B) green-red-emitting luciferases when an orange-emitting luciferase is 1; and (C) orange-red-emitting luciferases when a green-emitting luciferase is 1).

DISCLOSURE OF THE INVENTION

As a result of an intensive study for solving the above subjects, the present inventor has made a reporter gene construct capable of distinctively quantifying lights derived from 2 or more, preferably 3 or more and more preferably 4 or more luciferases (red-, orange-, green- and blue-emitting) based on the luciferases which emits different color lights (including red, orange, green and blue) or various luminescent substrates. According to the present invention, 2 or more, preferably 3 or more and more preferably 4 or more gene activities can be determined preferably simultaneously or at the same phase because

an emitted light intensity derived from each luciferase corresponds to a transcription activity of each promoter, i.e., the activity of the gene to which each promoter is originally linked. It is also possible to precisely determined because a luminescence wavelength is not changed due to a determining condition (pH, etc). For example, in one preferable embodiment of the present invention, a system for determining the transcription activities of multiple genes simply and highly quantitatively was made by making reporter gene constructs of a red-emitting luciferase and a green-emitting luciferase from a railroad worm and a green-emitting luciferase and an orange-emitting luciferase from *Rhagophthalmus ohba*, and simultaneously using luciferase reporter genes of *Renilla*, a marine ostracod, a luminescent dinoflagellate, a click beetle, aequorin and the like.

Furthermore, the present inventor has found that the transcription can be easily performed in mammalian cells for a luciferase which is scarcely expressed or is not expressed at all in the mammalian cells by (1) altering a cDNA sequence such that no additional transcription factor is bound and (2) changing a codon usage (bias of codon use frequencies) for insects to the codon usage for mammals in the cDNA sequence and further reducing restriction enzyme sites in the cDNA sequence because the many restriction enzyme sites limit an application of the cDNA.

The present invention provides the following polypeptide, gene, gene construct, mammalian cell, a method for screening drugs and a system for multiply determining the transcription activities of the promoters using the mammalian cells.

1. DNA encoding at least one luciferase selected from the group consisting a red-emitting luciferase and a green-emitting luciferase derived from a rail road worm and a green-emitting luciferase and an orange-emitting luciferase derived from *Rhagophthalmus ohba* stably expressed in mammalian cells, characterized in that (1) the DNA has no binding sequence for an additional transcription factor in the mammalian cells and has a codon usage for the mammal.

2. The DNA according to the above 1, characterized in that the mammal is human and the DNA has at least one nucleotide sequence selected from the group consisting of SEQ ID NOS:7, 10, 11 and 16.

3. A method for enabling the expression of DNA encoding a luciferase derived from a rail road worm or *Rhagophthalmus ohba* in mammalian cells, characterized by having

1) a step of altering a cDNA sequence such that no additional transcription factor is bound;

2) a step of changing a codon usage for insects to that for mammals in the cDNA sequence; and optionally

3) a step of altering the cDNA sequence with many restriction enzyme sites due to limited application at the use.

4. The method according to the above 3, characterized in that an amino acid sequence of the luciferase is not altered.

5. A polypeptide which is a luciferase with a maximum luminescence wavelength of 630 nm, represented by any of the followings:

(1) a polypeptide having an amino acid sequence of SEQ ID NO:4; and

(2) a polypeptide having one or more amino acid substitutions, additions or deletions in the sequence of SEQ ID NO:4.

6. The polypeptide according to the above 5, expressed in mammalian cells.

7. A gene construct incorporating one or two or more genes of luciferases which emit light whose wavelength does not substantially depend on a determining condition and maximum luminescence wavelength is 535 to 635 nm, to be stably expressible in mammalian cells.

8. The gene construct according to the above 7 incorporating 3 or more luciferase genes stably expressibly in mammalian cells by incorporating one or two or more genes of luciferases with a maximum luminescence wavelength of 460 to 520 nm together with one or two or more genes of luciferases which

emit light whose wavelength does not substantially depend on a determining condition and maximum luminescence wavelength is 535 to 635 nm.

9. The gene construct according to the above 7 wherein the above luciferase gene is a gene encoding at least one luciferase selected from the group consisting of a red-emitting luciferase and a green-emitting luciferase derived from a rail road worm and a green-emitting luciferase and an orange-emitting luciferase derived from *Rhagophthalmus ohba* stably expressed in mammalian cells.

10. The gene construct according to the above 7 comprising an element for promoting efficiency of translation and/or an element for stabilizing mRNA.

11. A gene construct capable of distinctively determining each light emitted from two or more luciferases, by incorporating one or two or more genes of the luciferases which emit light whose wavelength does not substantially depend on a determining condition and if necessary a gene of the luciferase which emits light whose wavelength is different and does not substantially depend on the determining condition under the control of different promoters.

12. An expression vector containing the gene construct according to any of the above 7 to 11.

13. Mammalian cells transformed with the gene construct according to any of the above 7 to 11 or the expression vector according to the above 12.

14. Mammalian cells stably expressibly incorporating two or more genes of luciferases which emit mutually distinct light whose luminescence wavelength does not substantially depend on a determining condition under the control of different promoters in the mammalian cells.

15. The mammalian cells according to the above 13 or 14 wherein two or more of the above luciferases have maximum luminescence wavelength of 535 to 635 nm and can emit with one substrate.

16. The mammalian cells according to the above 15 comprising a red-emitting luciferase gene from a rail road worm and further comprising at least two or more selected from the group consisting of a green-emitting luciferase gene from the rail road worm, a green-emitting luciferase gene from *Rhagophthalmus ohba*, an orange-emitting luciferase from *Rhagophthalmus ohba*, and a blue-emitting luciferase gene under the control of different promoters.

17. The mammalian cells according to the above 14 stably expressibly incorporating genes of three or more luciferases which emit mutually distinct light whose luminescence wavelength does not substantially depend on a determining condition under the control of different promoters in the mammalian cells.

18. The mammalian cells according to the above 14 having three or more luciferase genes under the control of different promoters wherein a first luciferase gene is under the control of a constantly expressed promoter, a second luciferase gene is under the control of a toxicity assessing promoter, and remaining one or more luciferase genes are under the control of a promoter subjected to assessment.

19. The mammalian cells according to the above 14 having three or more luciferase genes under the control of different promoters wherein a first luciferase gene is under the control of a constantly expressed promoter, a second luciferase gene is under the control of a pseudopromoter, and remaining one or more luciferase genes are under the control of a promoter subjected to assessment.

20. The mammalian cells according to the above 14 having 4 or more luciferase genes under the control of different promoters, wherein a first luciferase gene is under the control of a constantly expressed promoter, a second luciferase gene is under the control of a toxicity assessing promoter, a third luciferase gene is under the control of a promoter of a protein which accepts an external factor, and remaining one or more luciferase genes are under the control of a promoter subjected to assessment.

21. The mammalian cells according to the above 14 having 4 or more luciferase genes under the control of different promoters, wherein a first luciferase gene is under the control of a constantly expressed promoter, a second luciferase gene is under the control of a pseudopromoter, a third luciferase gene is under the control of a promoter of a protein which accepts an exogenous factor, and remaining one or more luciferase genes are under the control of a promoter subjected to assessment.

22. The mammalian cells according to the above 14 having two luciferase genes under the control of different promoters, wherein a first luciferase gene is under the control of a constantly expressed promoter, and a second luciferase gene is under the control of a toxicity assessing promoter.

23. The mammalian cells according to the above 14 having two luciferase genes under the control of different promoters, wherein a first luciferase gene is under the control of a constantly expressed promoter, and a second luciferase gene is under the control of a pseudopromoter.

24. A method for screening drugs including a step of culturing the mammalian cells according to any of the above 18 to 21 in the presence of a drug candidate compound in a medium of the mammalian cells, a step of quantifying an amount of the above luciferase in the presence or absence of the candidate compound, and a step of assessing an effect of the candidate compound on a promoter subjected to assessment, which is linked to at least one luciferase.

25. A system for multiply determining transcription activity of each promoter linked to each luciferase before and after a change of a culture environment by changing the culture environment of the mammalian cells according to any of the above 13 to 23, and assessing expressed amounts of two or more luciferases which emit mutually distinct light whose luminescence wavelength does not depend on a determining condition.

26. The system according to the above 23 for simultaneously determining expressed amounts of two or more luciferases.

27. The system according to the above 23 capable of determining expressed amounts of three or more luciferases.

The present invention will be illustrated in detail below.

Two or more luciferases in the present invention are required to emit light whose luminescence wavelength does not substantially depend on a determining condition (e.g., pH) because it is important to determine an emitted light intensity from two or more luciferases and calculate a relative ratio thereof.

As used herein, "the luminescence wavelength does not substantially depend on the determining condition" is that even if a pH, temperature, concentration or the like is changed, a variation of the maximum luminescence wavelength is 3 nm or less, preferably 2 nm or less, more preferably 1 nm or less and in particular, preferably 0.5 nm or less. If a changed amount of the maximum luminescence wavelength is within this range, when the expressed amounts of multiple luciferases are quantified by separating with a filter(s), it is preferable because a mutual ratio of the luciferases is scarcely changed.

As used herein, "two or more luciferases which emit mutually distinct light" means that it is possible to determine the ratio of emitted light intensities of the mutual lights using a filter (color filter, band pass filter, etc.). For example, for a red-emitting luciferase, a green-emitting luciferase from the rail road worm and an orange-emitting luciferase, a green-emitting luciferase from *Rhagophthalmus ohba*, it is possible to determine the ratio of emitted light intensities of mutual lights by using the filter to remove the green. To be capable of determining the ratio of emitted light intensities of the mutual lights, it is preferable to mutually separate the maximum luminescence wavelengths by usually 20 nm or more, preferably 30 nm or more, more preferably 40 nm or more and in particular, preferably 50 nm or more.

Preferable luciferases used in the invention include green-

to red-emitting (including mutants thereof, maximum luminescence wavelength: 535 to 635 nm, e.g., 540 to 630 nm) luciferases from the rail road worm, orange- to green-emitting (including mutants thereof, maximum luminescence wavelength: 530 to 600 nm) luciferases from the click beetle, and orange- to green-emitting (including mutants thereof, maximum luminescence wavelength: 550 to 590 nm) luciferases from *Rhagophthalmus ohba*, and the like. For example, in the case of the luciferases of the rail road worm, the red-emitting luciferase with a maximum luminescence wavelength of 622 nm and the green-emitting luciferase with a maximum luminescence wavelength of 545 nm have been known (US 2002/0119542), but the present inventor has identified that there exist many luciferases which emit lights with 540 to 635 nm in addition to these two. These luciferases can be all used. For example, the present inventor has confirmed that the red-emitting luciferase with a maximum luminescence wavelength of 622 nm (expressed in insects or *Escherichia coli*) from the rail road worm shifts the maximum luminescence wavelength to 630 nm when expressed in mammalian cells. This red-emitting luciferase with a maximum luminescence wavelength of 630 nm from the rail road worm was discovered for the first time by the present inventor.

When multiple luciferases are used, to distinctively determine each emitted light using the filter, it is desirable to mutually separate the maximum luminescence wavelength by 20 nm or more, preferably 30 nm or more, more preferably 40 nm or more and in particular, preferably 50 nm. By separating the maximum luminescence wavelength to this extent, the emitted light intensities of respective lights can be quantified simultaneously by using the filter between the maximum luminescence wavelengths, measuring a transmittance of each light before and after the filter, and converting.

For example, the maximum luminescence wavelengths of the luciferases in Fig. 20 are a red-emitting (630 nm), an orange (580 nm) and a green (550 nm), and these can be sufficiently separated.

In particular, when using the luciferases from the rail road worm and *Rhagophthalmus ohba* having the multiple luciferases whose maximum luminescence wavelengths are separated to some extent, it is possible to simultaneously quantify the emitted light intensities from the co-expressed multiple luciferases by the use of one luminescent substrate (e.g., a firefly luciferin can be used for the luciferases from the rail road worm, *Rhagophthalmus ohba* and the click beetle), and the ratio of the expressed amounts of promoters can be determined precisely. As the luciferase which emits the light whose luminescence wavelength does not depend on the determining condition (e.g., pH), it is possible to use *Renilla* luciferase, various luciferases of dinoflagellate (including a total sequence or luminescent domains such as Domains 1, 2 and 3; JP-2002-335961; Li L., Hong R., Hasting JW., Proc. Natl. Acad. Sci. USA (1997) 94, 8954), and marine ostracod luciferase by further combining. When the luciferases from the rail road worm, *Rhagophthalmus ohba* and the click beetle are used, the firefly luciferin can be used, and thus it is possible to reduce the background. The combination of the dinoflagellate luciferase with the luciferin is preferable because the background is low.

In one preferable embodiment of the present invention, it is possible to quantify the expressed amounts of at least three promoters with one luciferin by the use of the luciferases from the rail road worm, *Rhagophthalmus ohba* (e.g., the red-emitting luciferase from the rail road worm, the orange-emitting luciferase and the green-emitting luciferase from *Rhagophthalmus ohba*) (VR. Viviani, A. Uchida, N. Suenaga, M. Ryufuku & Y. Ohmiya: Thr-226 is a key-residue for bioluminescence spectra determination in beetle luciferases (2001)Biochem. Biophys. Res. Communi. 280, 1286-1291). It is also possible to quantify four or more by combining the blue-emitting luciferase (each luciferase of *Renilla*, the dinoflagellate or the marine ostracod). By successfully setting the filters, it is possible to analyze multiple expressions between 540 to 635 nm (green to red-

emitting), and preferably between 540 to 630 nm. Further, one more can be added by a blue-emitting luciferase whose substrate is different. Therefore, as the simultaneous determination of the luciferases, it is possible to simultaneously quantify three or more when the same luciferin is used, and four or more when the different luciferins are used.

Conventionally, as the luciferases expressible in the mammalian cells, the *Renilla* luciferase and the firefly luciferase have been known. However, the color of the light emitted from the firefly luciferase varies from green to yellow depending on the pH of a cell lysed solution. Therefore, when the expressed amounts of two or more luciferases are compared, there has been a drawback that an accuracy is lacked. The blue luminescence derived from the *Renilla* luciferase is desirable in that its luminescence wavelength does not substantially depend on the determining condition, but in the determination system in which the firefly luciferase is combined, it is necessary to separately perform both the quantification using the firefly luciferin and the quantification using the *Renilla* luciferin. Thus, there has been a drawback that simplicity and accuracy are lacked.

The present inventor focused on the luciferase from the rail road worm as the luciferase other than *Renilla* luciferase and the firefly luciferase, and attempted to express this protein in the mammalian cells, but could not express the luciferase from the rail road worm in the mammalian cells using usual expression systems. This is believed to be a reason why no luciferase other than *Renilla* luciferase and the firefly luciferase has been expressed in the mammalian cells, particularly human cells.

According to findings until now of the present inventor, in one preferable embodiment of the invention, what really matters upon practical application of the rail road worm luciferase, the *Rhagophthalmus ohba* luciferase and the marine ostracod luciferase is that a rail road worm luciferase gene, the *Rhagophthalmus ohba* luciferase gene and the marine ostracod luciferase gene are

stably transcribed and stably translated. In a technique used in Example of the present invention, it has been proven that the practical application becomes possible by stabilizing transcribed mRNA and increasing a number of translation frequency. That is, in this case, it has become possible for the first time that the luciferase gene from the rail road worm is expressed in the mammalian cells by inserting a globulin intron to prolong a lifespan of mRNA and inserting a Kozak sequence to increase the translation frequency.

Further techniques in the preferable other embodiments of the invention include, for example, changing the cDNA sequence from the codon usage (bias of codon use frequency) for insects to that for mammals for increasing copy numbers of mRNA, changing the cDNA sequence such that no additional transcription factor is bound, and changing the cDNA sequence with many restriction enzyme sites because the application of such a sequence is limited. Such techniques were useful for the expression of the luciferase from the rail road worm and the *Rhagophthalmus ohba* luciferase in the mammalian cells. In particular, the change to the codon usage (bias of codon use frequency) for the mammals and the change of cDNA sequence such that no additional transcription factor is bound are more useful.

The change of the cDNA sequence can be performed by considering the following order 1) to 4) sequentially:

1) the amino acid sequence of the luciferase is not changed as possible (preferably not changed at all);

2) subsequently, the cDNA sequence is changed such that no additional transcription factor is bound;

3) further, the codon usage for the insects is changed to that for the mammals in the cDNA sequence; and

4) if necessary, the cDNA sequence is changed to reduce the restriction enzyme sites.

In the above, the expression of the luciferase from the rail road worm and the *Rhagophthalmus ohba* luciferase was described, but the luciferases from other organisms such as a

click beetle are believed to similarly express.

As used herein, the "luciferase" encompasses a light-emitting enzyme group such as luciferase which catalyzes a luciferin photochemical reaction, and also includes those such as aequorin. A protein having a luminescence action obtained by changing a luciferin structure, whose catalysis (action where the luciferin is oxidized to convert a light-emitting substance) is weak can be included in the luciferase of the present invention as long as its luminescence wavelength does not substantially depend on the determining condition (e.g., pH).

As the luciferases, it is desirable to combine two or more luciferases which emit the light with the same luminescent substrate. As preferable luciferases whose luminescence wavelength is not substantially changed by the determining condition and which emit the light with the same substrate, a red-emitting luciferase from the rail road worm and a green-emitting luciferase from the rail road worm or other luciferases from the rail road worm having the luminescence wavelength in the range of about 540 to 635 nm, preferably about 540 to 630 nm, further a green-emitting luciferase from *Rhagophthalmus ohba* and an orange-emitting luciferase from *Rhagophthalmus ohba* are preferably exemplified. In addition to them, the luciferases (about 530 to 600 nm) from the click beetle are also exemplified. In particular, the red-/green-emitting luciferases from the rail road worm and the orange-/green-emitting luciferases from *Rhagophthalmus ohba* are convenient for multiply quantify the transcription activities of promoters because emitted light intensities are almost the same when amounts of the luciferases are the same.

In the present invention, the mammals include human, cattle, horse, sheep, monkey, swine, mouse, rat, hamster, guinea pig, rabbit and dog, and is preferably the human.

It is preferable that at least two luciferase genes emit different color lights with the same substrate and their intracellular lifespan be similar. In this respect, the red-

/green-emitting luciferases from the rail road worm and the orange-/green-emitting luciferases from *Rhagophthalmus ohba* are preferable. In particular, the red-emitting luciferases from the rail road worm and the orange-/green-emitting luciferases from *Rhagophthalmus ohba* are preferable.

Furthermore, for the quantification of each emitted light color by a simple apparatus, it is preferable to be capable of separate by a filter (s) different emitted light colors of at least one, preferably at least two luciferases whose luminescence wavelengths used in the invention are not changed by the determining condition (e.g., pH) and the other luciferase for standardizing the above luciferases. For example, as shown in Fig. 5, the red-/green-emitting luciferases from the rail road worm is preferable because they can be easily separated using the filter. Furthermore, the combination of the red-/green-emitting luciferases from the rail road worm with the luciferase (maximum luminescence wavelength: 474 to 480 nm) derived from *Renilla* or the dinoflagellate is particularly preferable because the emitted lights can be easily separated using two filters as shown in Fig. 10. The lights emitted from the red-emitting luciferases from the rail road worm and the orange-/green-emitting luciferases from *Rhagophthalmus ohba* can be mutually separated using two filters (Fig. 19).

The luciferases from the rail road worm as in the above have been known to express in *Escherichia coli*, but no expression thereof in the mammalian cells, particularly human cells has been known. In fact, even when the expression of the luciferases (red-emitting, green-emitting) was attempted in the human cells, the expression could not be induced using an SV40 or CMV promoter alone which is a representative expression promoter in the mammalian cells as shown in Fig. 2 and Example 1. In the case of *Rhagophthalmus ohba* luciferases, an expression level is too low to apply practically in the sequence known publicity, but the mutants of the present invention have the expression levels of 44 times (green) and 57 times (orange) compared to wild-type

luciferase, and have significant practicability. In the preferable embodiment of the invention, an expressed amount of the wild-type is insufficient because the expressed amount of the luciferase with particular color is evaluated in luminescence spectra using the filter(s).

Gene sequences of the luciferases (red-emitting, green-emitting) from the rail road worm are disclosed in US 2002/0119542-A1. A red-emitting luciferase gene (having an error) in US 2002/0119542-A1 is shown in SEQ ID NO:5. Correct nucleotide sequences of the luciferases from the rail road worm are shown in SEQ ID NO:1 (green-emitting luciferase gene) and SEQ ID NO:3 (red-emitting luciferase gene), and correct amino acid sequences are shown in SEQ ID NO:2 (green-emitting luciferase gene) and SEQ ID NO:4 (red-emitting luciferase gene).

As the luciferase gene of the present invention, intact wild-type or mutant luciferase genes can be used, and it is possible to use DNA capable of hybridizing with the luciferase gene under a stringent condition and DNA encoding a polypeptide having one or more amino acid substitutions, additions, deletions or insertions in the luciferase and having a luciferase activity as the luciferase gene.

In one preferable embodiment, the present inventor has found by examining various expression systems that it is important for stable expression of the luciferase in the mammalian cells to introduce an element for promoting efficiency of the translation and/or an element for stabilization of mRNA into a gene construct. As the element for promoting the efficiency of the translation, Kozak sequence (Ko) and the like are exemplified. As the element for the stabilization of mRNA, β-globin intron II and the like are exemplified. To stably express the luciferase in the mammalian cells, in particular, a partial structure of (globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase) is preferable. It has been also confirmed that it is preferable for stable expression of the luciferase in the mammalian cells to change the codon usage (bias of codon use

frequency) for the insects to that for the mammals and change the cDNA sequence such that no additional transcription factor is bound.

In one preferable embodiment, the gene construct of the invention can contain a luciferase gene, a promoter, the element for promoting the efficiency of the translation and/or the element for the stabilization of mRNA upstream of the gene, and further can contain an enhancer, IRES, SV40pA, a drug resistant gene (Neo^r, etc.) and the like.

Examples of the preferable gene construct of the present invention will be shown below.

(1) (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(-/green-emitting luciferase)-(SV40 poly A sequence)

(2) (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(IRES)-(Neo gene)-(SV40 poly A sequence)

The gene construct of the invention may be directly introduced into the mammalian cells, but it is preferable to incorporate in a vector (e.g., including a plasmid and a viral vector) to introduce into the mammalian cells. When multiple luciferases are incorporated expressibly in a gene construct, one gene construct or expression vector may be introduced into the mammalian cells, and when one luciferase is incorporated in one gene construct, multiple gene constructs or expression vectors may be simultaneously or sequentially introduced into the mammalian cells according to the standard method.

As combination of genes desirably determined simultaneously by the system of the present invention,

-clock genes (Per gene, Clock gene, BMAL gene, etc.)

-cancer genes (oncogene, tumor suppressing gene, mitosis marker gene, etc.)

-genes involved in diseases (pathology corresponding gene, life and death sensitive apoptosis gene, hormone gene, etc.)

-constantly expressed genes (actin gene, GAPDH (glyceraldehyde

phosphate dehydrogenase) gene, monkey-derived SV40 virus gene, etc) and the like are exemplified.

The following application can be performed in the present invention.

(1) Primary screening: It is important to simultaneously obtain 3 or more information on the assumption that many specimens are exhaustively analyzed. Obviously multiple combinations are thought. Considering drug discovery, it is necessary to evaluate not only positive points but also negative toxic points for effects of the drug. Furthermore, changes of two genes at a transcriptional level reflect a status of a cell itself, and thus, it is preferable to use a constantly expressed promoter which indicates the status of the cell as a control. Therefore, in the drug discovery screening, the following combinations are exemplified.

In tables 1 and 2, -/blue-/green-emitting luciferases are only exemplified, and it goes without saying that the other luciferases including the orange-emitting luciferase or the combinations thereof can be used. In particular, the combination of red-/orange-/green-emitting luciferases from the rail road worm and *Rhagophthalmus ohba* is particularly preferable because they can be simultaneously determined with the firefly luciferin.

The luciferases with various color can be optionally selected.

Table 1

Drug discovery screening

Subject promoter + green-emitting luciferase	Evaluation of drug effect
Toxicity evaluation promoter(apoptosis-related) + blue-emitting luciferase	Evaluation of drug safety
Constantly expressed promoter + red-emitting luciferase	Evaluation of cell condition
Green-/red-emitting: standardization of drug effect Blue-/red-emitting: standardization of safety	

In this case, the toxicity evaluation and the constant expression are controls of the promoter subjected to the drug

evaluation, and thus it is also useful to construct in one vector. A cell itself in which this vector has been incorporated is a basic cell for screening.

Table 2

Search of target promoter sequence

Unspecified promoter (sequence group whose effect is unknown on promoter library) + Green-emitting luciferase	Evaluation of drug effect
Pseudopromoter sequence (random sequence or nonsense sequence) + Blue-emitting luciferase	Evaluation of drug safety
Constantly expressed promoter + red-emitting luciferase	Evaluation of cell condition
Green-/-emitting: standardization of promoter effect Blue-/red-emitting: standardization of pseudo information	

In this case, the pseudopromoter and the constantly expressed promoter are controls of the promoter subjected to the screening, and thus it is also useful (not essential) to construct in one vector. A cell itself in which this vector has been incorporated is a basic cell for screening.

The combination of -/orange-/green-emitting can accomplish the screenings represented in Tables 1 and 2 using one substrate. That is, the blue-emitting luciferase is substituted with the orange-emitting luciferase. In this case, the determination can be performed using one substrate, and the determining method is simpler. For determining the blue-emitting luciferase, it is necessary to lyse cells, but the firefly luciferin permeates into living cells with a concentration gradient and emits the light, and thus it is possible to determine three emitted lights in the living cells. Therefore, this method is characterized in that the screening can be performed without lysing the cells.

Meanwhile, the combination of red-/orange-/green-/blue-emitting has an advantage that an external factor such as environmental disruptors can be simultaneously evaluated, and can determine a change of transcription activities of multiple genes in the cell affected by the external factor. For example,

monitoring of the expression of receptor which directly captures the external factor is included.

Table 3

Unspecified promoter (sequence group whose effect is unknown on promoter library) + Green-emitting luciferase	Evaluation of external factor effect
Pseudopromoter sequence (random sequence or nonsense sequence) + Blue-emitting luciferase	Evaluation of external factor safety
Promoter sequence of accepting protein of external factor + Orange-emitting luciferase	Evaluation of accepting process of external factor
Constantly expressed promoter + red-emitting luciferase	Evaluation of cell condition
Green-/red-emitting: standardization of promoter effect, Blue-/red-emitting: standardization of pseudo information, Orange-/red-emitting: standardization of external factor acceptance, Green-/orange-emitting: evaluation of acceptance and activation	

In this case, a protein which accepts the external factor, a protein affected thereby, and further the safety of the cell itself can be evaluated, and the information which the external factor gives to the cell can be precisely evaluated by standardizing them with the control of the protein of the constantly expressed promoter. Thus, it is also useful (not essential) to construct in one vector. A cell itself in which this vector has been incorporated is a basic cell for screening.

Examples of the primary screening are shown in Fig. 11.

(2) Secondary screening: It is important to obtain 3 or more information on the assumption that the focused drug effects and promoter signals are evaluated. In the drug discovery, multiple effects of the drug are often assumed. It is also important to know a gene which indicates the change of cell condition, transient effects of the drug (e.g., toxicity, shock response, etc.), and actual effects. For example, as shown in Tables 3 and 4, evaluation systems of clock-related drug effects can be exemplified.

Table 3

Evaluation system of clock-related drug effects

Drug detection promoter (e.g., toxicity, shock response, etc.) + Green-emitting luciferase	Evaluation of transient effect of drug
Diurnally varying promoter (sequence of BMAL or Per gene) + Blue-emitting luciferase	Evaluation of biological clock
Drug corresponding promoter + Red-emitting luciferase	Evaluation of intracellular effects of drug
Blue-/green-/red-emitting: temporal axis evaluation of drug	

Table 4

Evaluation system of clock-related drug effects

Drug detection promoter (e.g., toxicity, shock response, etc.) + Green-emitting luciferase	Evaluation of transient effect of drug
Diurnally varying promoter (sequence of BMAL or Per gene) + Orange-emitting luciferase	Evaluation of biological clock
Drug corresponding promoter + Red-emitting luciferase	Evaluation of intracellular effects of drug
orange-/green-/red-emitting: temporal axis evaluation of drug	

In particular, as in the above, it is possible to determine three emitted lights in the living cells using one substrate. Therefore, the method is characterized in that the drug effect can be evaluated according to the temporal axis without lysing the cells.

A series of operations is performed for the same cells, and thus the drug effect on the combination (history)of the multiple operations can be evaluated.

An example of the secondary screening is shown in Fig. 12.

As in the above, by preferably simultaneously evaluating expressed amounts of 2 or more, particularly 3 or more or 4 or more promoters, when actions for one promoter are evaluated, it is possible to standardize an activity, toxicity and the like or

standardize pseudo information.

Furthermore, when a phenomenon where the expressions of multiple genes are intricately related in the mammal is elucidated, the system of the present invention is extremely useful.

In the particularly preferable embodiment of the invention, the method/system for simultaneously quantifying three or four gene transcription activities using the red-emitting luciferase gene, the green-emitting luciferase gene from the rail road worm, the green-emitting luciferase gene, the orange-emitting luciferase gene from *Rhagophthalmus ohba*, and the blue-emitting luciferase gene is provided. By the use of this system, it is possible to simultaneously determine multiple transcription activities in the cells. It is possible to utilize them for the treatment/examination of pathology and the new drug development.

At that time, color identification is performed, and the multiple transcription activities in the cells can be simultaneously determined by determining the luminescence activity using the filters specified for the red-, green-, orange- and blue-emitting. Much information can be simultaneously elicited for the change in the cells, whose information has been conventionally difficult to obtain from change information of one transcription activity, and can be utilized for the treatment of various diseases and the new drug development.

In the present invention, the mammalian cells having two luciferase genes under the control of distinct promoters (1) wherein a first luciferase gene is under the control of the constantly expressed promoter and a second luciferase gene is under the control of the toxicity assessing promoter or (2) wherein the first luciferase gene is under the control of the constantly expressed promoter and the second luciferase gene is under the pseudopromoter according to claim 14 are useful as intermediate cells for producing the mammalian cells for drug screening by further introducing the gene construct in which one or more luciferase genes are incorporated under the control of

promoters subjected to the evaluation in these mammalian cells.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be illustrated in more detail with reference to the following Examples, but it goes without saying that the invention is not limited to the Examples.

Example 1

A green-emitting luciferase gene and a red-emitting luciferase gene (SEQ ID NO:1, 3) from a rail road worm are expressed in *Escherichia coli*, but the expression thereof can not be induced in mammalian cells using an SV40 or CMV promoter alone which is a representative expression promoter in mammals. Thus, a construct in which Kozak sequence and β-globin intron II which stabilize the gene expression were inserted, and further chicken β-actin promoter and CMV enhancer were selected was ligated to the red- or green-emitting luciferase gene to make a gene structure, and an enzyme activity was determined. (Fig. 2). This was compared with a gene structure in which the luciferase gene had been inserted downstream of the SV40 promoter, CMV promoter or CAG promoter. Cultured fibroblast cells, NIH3T3 cells were transfected with each gene using Lipofectamine, and a luminescence activity after 24 hours in the cells was determined (Fig. 2). For determining the luminescence activity, a luminescent substrate mixed solution (supplied from Toyo B-Net Co., Ltd.) and AB-2000 were used as a substrate and as a luminescence determining apparatus supplied from ATTO Corporation, respectively. To 50 μL of a cell extract solution, 50 μL of PicaGene was added. As a result, the highest activity was obtained in the cells into which the (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(SV40 poly A sequence) gene had been introduced. The secondarily highest activity was obtained in the cells into which the construct in which (IRES)-(Neo gene)-(SV40 poly A sequence) had been inserted in place of (SV40 poly A sequence) had been introduced. However, the SV40 promoter or the

CMV promoter alone elicited almost no activity. But, the activity elicited by the (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(red-/green-emitting luciferase)-(IRES)-(Neo gene)-(SV40 poly A sequence) gene was about 500/1, and the activity elicited by the (CMV promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(SV40 poly A sequence) gene was about 10/1 based on the activity elicited by the (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(IRES)-(Neo gene)-(SV40 poly A sequence) gene. Therefore, it has demonstrated that it is preferable to insert (β-globin intron II)-(Kozak sequence) upstream of the enzyme gene, which is a region which does not directly affect the transcription activity in order to stably express the green-emitting luciferase gene from the rail road worm and determine the gene transcription activity. This is believed to attribute to the promotion of translation efficiency due to the Kozak sequence and the stabilization of mRNA due to the β-globin intron II. It has been demonstrated that the promotion of efficiency of and the stabilization of the transcript including the luciferase gene are keys for practical application.

Example 2

Luminescence spectra of the red-emitting luciferase gene and the green-emitting luciferase gene from the rail road worm expressed in the mammalian cells were analyzed. To 15μL of an extract solution of cells into which the (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(SV40 poly A sequence) genes which exhibited the highest activity had been introduced, 15 μL of PicaGene was added, and the luminescence spectrum was determined using a weak luminescence spectrum measuring apparatus supplied from ATTO Corporation. Fig 3 shows the luminescence spectra when the spectrum was expressed singly, the maximum luminescence wavelengths of 630 nm and 550 nm were observed in the red-emitting luciferase and the green-emitting luciferase,

respectively. These spectra were not affected by pH and a surrounding solutions, and were not changed at all.

Example 3

Lifespan in the cells of the red-emitting luciferase gene and the green-emitting luciferase gene from the rail road worm expressed in the mammalian cells was evaluated. The cells into which the (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-emitting, green-emitting luciferase)-(IRES)-(Neo gene)-(SV40 poly A sequence) genes had been introduced were used. Cultured fibroblast cells, NIH3T3 cells were transfected with the above luciferase gene to be expressed in the cells by a lipofection method. Forty-eight hours after the transfection, the medium was replaced with a medium containing 100 μM of a protein synthesis inhibitor, cycloheximide, and the cells were cultured for 30 min. Subsequently, the luminescence activity was determined with time by the same method as that in Example 1. As a result, for both red and green-emitting luciferases, the activity was reduced in the similar time course, and a half life of each enzyme in the cells was about 3.5 hours (Fig. 4).

Example 4

The red-emitting luciferase gene and the green-emitting luciferase gene from the rail road worm, the (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(SV40 poly A sequence) genes were co-expressed in the cultured fibroblast cells NIH3T3. The luminescence spectrum of the red-emitting luciferase gene and the green-emitting luciferase gene from the rail road worm in a cell extract solution obtained by lysing the co-expressing cells was determined by the same technique as that in Example 2. Fig. 5 shows the luminescence spectrum of the co-expressing cells. Two peaks were observed because the red-emitting luciferase and the green-emitting luciferase emit lights. This is a result of simultaneously determining two gene transcription activities. When these luminescence activities is determined by a luminometer

using a photomultiplier, a total sum of the luminescence activities of two red and green-emitting luciferase genes from the rail road is observed. Thus, to determine only the luminescence activity of the green-emitting luciferase, the light of the red-emitting luciferase was cut off. Evaluating from the luminescence spectrum, a cut off filter of light wavelengths represented by a dot line in Fig. 5 was selected. By the use of this filter, 8% of the green-emitting luciferase activity and 76% of red-emitting luciferase activity can be detected, and by converting it is possible to evaluate emitted light intensities from the red- and green-emitting luciferases and abundance thereof.

Example 5

To 50 μL of a cell extract solution containing the red-emitting luciferase and the green-emitting luciferase, 50 μL of PicaGene was added, and the luminescence activity was measured with one min intervals using a dish type luminometer AB2500 supplied from ATTO Corporation to yield luminescence reaction curves as shown in Fig. 6. The activity was not stabilized within 5 min after the start of the reaction, but both activities were stabilized after 6 min. Thus, when the luminescence activity in the cells in which the red-emitting luciferase gene and the green-emitting luciferase gene had been co-expressed was measured, the activity was measured at a time zone at which the luminescence reaction was stable. A measuring procedure is as follows: 1) the emitted light intensity is measured without a filter (color filter R54 type supplied from Hoya Corporation) (luminescence activities of red- and green-emitting luciferases); 2) the filter (color filter R54 type supplied from Hoya Corporation) determined in Example 4 is inserted in the luminometer, the emitted light intensity is measured to make it the luminance activity of the green-emitting luciferase; and 3) the luminescence activity of the red-emitting luciferase is calculated by converting a transmittance of the filter (color filter R54 type supplied from Hoya Corporation).

Example 6

It was examined in a model experiment whether the red-emitting luciferase and the green-emitting luciferase at different abundance ratio can be quantified by the procedure determined in Example 5. In Fig. 7, for samples in which the abundance ratio of the red-emitting luciferase and the green-emitting luciferase had been changed, 1) total intensities of emitted light were measured; 2) only the green-emitting luciferase was measured, and 3) the amounts of the red-emitting and green-emitting luciferases were quantified. As a result, it has been demonstrated that the luminescence activity is changed in a linear relationship with the abundance ratio thereof. This indicates that the amounts of the red-emitting luciferase and the green-emitting luciferase which have shown different expressed amounts in the cells can be quantified by the luminometer to which the color filter (color filter R54 type supplied from Hoya Corporation) was inserted.

Example 7

To examine an availability of the present system, the gene transcription activities of two clock genes were measured, and standardized using simultaneously a promoter which exhibited the constant gene transcription activity as the third gene transcription activity. Specifically, the NIH3T3 cells were co-transfected with an (E54), an element linking an E-box 3, 4, 5 in mouse Per promoter-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-emitting luciferase)-(SV40 poly A sequence) gene and an REV-ERV/ROR element 1,2 (RORE) in mouse BMAL1 promoter-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(green-emitting luciferase)-(SV40 poly A sequence) gene, and a blue-emitting luciferase vector for the standardization (phRL-TK, Promega) together with human BMAL1, human CLOCK, and mouse ROR-4 expression vector. After 24 hours, the cells were lysed, and luciferase luminescence wavelengths in the cells were analyzed using a spectrometer. As a result, the luminescence wavelengths from these two luciferases were detected,

and these showed the same luminescence spectrum as that when the individual luciferase alone was expressed. Thus, the luminescence activity of the red- and green-emitting luciferases was measured. The transcription activities obtained by further standardizing these activity values with the activity value of the blue-emitting luciferase are shown in Fig. 8. In separate experiments, it has been known that when BMAL1 and CLOCK proteins are expressed in the cells, the element (E54) promoter linking the E-box 3, 4, 5 is activated and an (RORα) promoter is inactivated whereas when the mouse ROR-4 is expressed in the cells, the (RORα) promoter is highly activated. The activities of the red- and green-emitting luciferases simultaneously measured in the present experiment quantitatively show the transcription activity difference of (E54) promoter and (RORα) promoter.

Example 8

The red-emitting luciferase gene and the green-emitting luciferase gene from the rail road worm, (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(SV40 poly A sequence) genes and the blue-emitting luciferase vector (phRL-TK, Promega) were co-expressed in the cultured fibroblast cells NIH3T3. The co-expressing cells were lysed, and the luminescence spectrum of the red-emitting luciferase and the green-emitting luciferase from the rail road worm and the blue-emitting luciferase from *Renilla* in a cell extract solution was measured by the same technique as that in Example 2. Fig. 9 shows the luminescence spectrum of the co-expressing cells. Three peaks emitted from the red-, green- and blue-emitting luciferases were observed, and heights of the peak reflect heights of respective promoter activities. The transcription activities of three genes can be evaluated by converting to the intensity of emitted red, green or blue light using a an emitted light intensity determining apparatus with filters capable of identifying the emitted light colors.

Example 9

The NIH3T3 cells were co-transfected with the red-emitting

luciferase gene and the green-emitting luciferase gene from the rail road worm, (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)-(red-/green-emitting luciferase)-(SV40 poly A sequence) genes by lipofection. After culturing for 16 hours, the medium was replaced with a medium containing 100 nM dexamethasone, and the cells were cultured for 2 hours. Subsequently, the medium was replaced with a medium containing 100 μM firefly luciferin, and the luminescence activity of the red- and green-emitting luciferases was continuously measured using the dish type luminometer AB2500 supplied from ATTO Corporation. Fig. 10 shows the result of continuously measuring the transcription activity. If using a continuous emitted light intensity determining apparatus which identifies the emitted light colors, it is possible to continuously measure two transcription activities.

Example 10

To stably express in the mammalian cells, the sequence of the red-emitting luciferase gene was designed with keeping the followings in mind. By (1) the changes of 34 transcription factor binding sites (48 DNA sequences) (Table 4); (2) the changes of 279 DNA sequences for making the codon use frequency close to the mammalian use frequency (Table 5); and (3) the changes of 15 common restriction enzyme sites (4 in 45 DNA sequences are the same as those of the transcription factor binding sites) (Table 6), the sequence of SEQ ID NO:7 was designed and a construct (SEQ ID NO:7) was artificially made. This sequence has 77.5% homology with the wild-type red-emitting luciferase gene (SEQ ID NO:3) from the rail road worm and 82.8% homology with the red-emitting luciferase mutant described in WO 2003/016839 (Figs. 13 and 14).

Table 4

Predicted transcription factor	Position number	Mutant sequence (Italic means a mutated part)	
Octamer-binding factor 1	89 - 103	(A99T)	cagcaggactacaattatatcaatcattat ataaatattc*t*tat a ttactgacggaataatcgatgcccatacca
Pit1, GHF-1 pituitary specific pou domain transcription factor	91-101	(T96C) (A99G)	gcaggactacaattatatcaatcattatat aaata*c*tc*g*ta ta ttactgacggaataatcgatgcccatac
Myf5 myogenic bHLH protein	164 - 178	(C168A) (C171T)	caatgaagtaatatcatatgctcaaatatt tgaaac*a*ag*t*tgcc gct tggcagttagtctagaaaaatatggcttgg
E2F, involved in cell cycle regulation, interacts with Rb p107 protein	186 - 200	(A195G)	aatatttgaaaccagctgccgcttggcagt tagtctaga*g*aaat a tggcttggatcataacaatgttgtggcaat
cellular and viral TATA box elements	268 - 284	(T276C) (T277C)	gaaaacaacatacacttttttggcccttta attgctg*cc*ctata cca aggaatacaatggcaacatcaaatgatat
Ikaros 3, potential regulator of lymphocyte differentiation	281 - 293	(A288G)	Actttttggcccttaattgctgctttat accaagg*g*atacc aatggcaacatcaaatgatatgtacacaga
cellular and viral CCAAT box	332 - 342	(T336C)	catcaaatgatatgtacacagaaagggaga tgat*c*ggccat tt gaatatatcgaaaccatgccttatgttt
Mammalian C-type LTR TATA box	424 - 440	(C426T) (T429C)	tttattctgaaagtacaaaaacatctagat tt*t*ct*c*aaaaaagt cat agtcattgatagtatgtacgatatcaatgg
TCF/LEF-1, involved in the Wnt signal transduction pathway	484 - 500	(C489T)	atgtacgatatcaatggcgttgaatgcgta tttag*t*tttgtttc acg ttatactgatcacgcctttgatccagtgaa
X-box-binding protein 1	491 - 505	(T501G)	atatcaatggcgttgaatgcgtatttagct ttgtttcacg*g*tat a ctgatcacgcctttgatccagtgaaattta
TCF/LEF-1, involved in the Wnt signal transduction pathway	511 - 527	(C516G) (T519C)	gtatttagctttgtttcacgttatactgat cacgc*g*tt*c*gatcc agt gaaatttaacccaaaagagtttgatccctt
Hox-1.3, vertebrate homeobox protein	562 - 578	(A570G) (T571C)	tttaacccaaaagagtttgatcccttggaa agaaccgc*gc*taat tat gacatcatctggaacaactggattgcctaa
COMP1, cooperates with myogenic proteins in multicomponent complex	593 - 613	(A600C) (T601C)	gaaccgcattaattatgacatcatctggaa caactgg*cc*tgcct aaagggg tagtaataagccatagaagtataactatаа
Prostate-specific homeodomain protein NKX3.1	626 - 638	(A630G)	ctggattgcctaaaggggtagtaataagcc atagg*a*gtataac tataagattcgtccatagcagtgatcccat
POU factor Brn-2 (N-Oct 3)	817 - 833	(A822C)	aagaaatttgagggcgaattcttcttaaaa accat*c*caaaatta caa aatcgcttctattgtagttcctcctccaat
Pu.1 (Pul20) Ets-like transcription factor identified in lymphoid B-cells	844 - 860	(T828C)	agggcgaattcttcttaaaaaccatacaaa a*c*tacaaaatc gc ttctattgtagttcctcctccaattatg
Hox-1.3, vertebrate homeobox protein	880 - 896	(A888T) (T889C)	gttcctcctccaattatggtatatttggct aaaagtcc*t*c*t*agt cga tgaatacaatttatcgagcttaacggaaat
transcriptional repressor CDP	903 - 919	(T907C) (A909G)	tttggctaaaagtccattagtcgatgaata caat*g*t*g*tcgagc ttaacggaaattgcttgtggagggtctcct
complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2	951 - 963	(T957C)	ggaaattgcttgtggagggtctcctttagg aagaga*c*atcgca gataaagtagcaaagagattgaaagtacat
TCF/LEF-1, involved in the Wnt signal transduction pathway	967 - 983	(A975C)	gggtctcctttaggaagagatatcgcagat aaagtagc*c*aagag att gaaagtacatggaatcctacaaggatatgg
Prostate-specific homeodomain protein NKX3.1	1036 - 1048	(T1044G)	ggatatggattaaccgaaacctgcagcgct ctaatact*g*agcc ccaatgatcgagaacttaaaaaaggtgcaa
transcriptional repressor CDP	1049 - 1065	(T1053C) (C1057A)	ccgaaacctgcagcgctctaatacttagcc ccaa*c*gat*a*gagaa ctt aaaaaaggtgcaattggaacgcctatgcca
Ribonucleoprotein associated zinc finger protein MOK-2 (human)	1066 - 1086	(A1071G)	ctaatacttagccccaatgatcgagaactt aaaaa*g*ggtgcaat tggaacg cctatgccatatgttcaagttaaagttata
Octamer-binding factor 1, POU-specific domain	1158 - 1170	(A1161G) (A1164T)	tgggaaggcgctaggaccaagagaaaaagg cga*g*at*t*tgcttc aaaagtcaaatgcttatgaaaggatatcac
Ecotropic viral integration site 1 encoded factor	1182 - 1198	(A1191G) (A1194C)	aaaaggcgaaatatgcttcaaaagtcaaat gcttatgaa*g*gg*c*t atc acaacaatccgcaagcaactcgtgatgctc
Nuclear factor Y (Y-box binding factor)	1236 - 1250	(T1242G)	tccgcaagcaactcgtgatgctcttgacaa agatggg*g*tggcttc a tactggggatcttggatattacgacgaaga
Prostate-specific homeodomain protein NKX3.1	1309 - 1321	(A1314G)	gacagatttatctatgtagttgatcgattg aaaga*g*cttatta aatataaaggatatcaggttgcgcctgctg
Special AT-rich sequence-binding protein 1, predominantly expressed in thymocytes, binds to matrix attachment regions (MARs)	1314 - 1330	(T1317C) (T1320C)	atttatctatgtagttgatcgattgaaaga act*c*at*c*aaatata aag gatatcaggttgcgcctgctgaactggaaa
Octamer-binding factor 1	1373 - 1387	(T1377C)	cgcctgctgaactggaaaatctgcttttac aaca*c*ccaaatatt t ctgatgcgggtgttattggaattccggacg
POU factor Brn-2 (N-Oct 3)	1379 - 1395	(A1380T)	ctgaactggaaaatctgcttttacaacatc c*t*aatatttctgat gcg ggtgttattggaattccggacgaatttgct
Octamer-binding factor 1	1399 - 1413	(T1401C)	ttacaacatccaaatatttctgatgcgggt gt*c*attggaattcc g gacgaatttgctggtcaattaccttccgcg
Binding site for a Pbx1/Meis1 heterodimer	1422 - 1438	(A1431G)	tgcgggtgttattggaattccggacgaatt tgctggtca*g*ttac ctt ccgcgtgtgttgtgttagagcctggtaaga
GATA-binding factor 2	1548 - 1560	(A1551C) (T1554C)	aactaaacatcttcgaggcggtgtcgtatt tat*c*ga*c*agtatt ccaaaaggcccaacaggaaaactcatgaga
Cart-1 (cartilage homeoprotein 1)	1624 - 1640	(T1636C)	gaactccgtgcaatatttgcccgggaacag gcaaaatcaaaa*c*t ata a

Table 5

Amino Acid	Codon	RED complete #	RED complete %	RED-mutant #	RED-mutant %
Met	ATG	14	100.0	14	100.0
Trp	TGG	1	100.0	1	100.0
Glu	GAA	26	83.9	3	9.7
	GAG	5	16.1	28	90.3
Phe	TTT	19	76.0	7	28.0
	TTC	6	24.0	18	72.0
Asp	GAT	25	83.3	16	53.3
	GAC	5	16.7	14	46.7
Cys	TGT	3	33.3	5	55.6
	TGC	6	66.7	4	44.4
His	CAT	12	80.0	0	0.0
	CAC	3	20.0	15	100.0
Gln	CAA	12	80.0	0	0.0
	CAG	3	20.0	15	100.0
Asn	AAT	13	65.0	2	10.0
	AAC	7	35.0	18	90.0
Tyr	TAT	17	70.8	9	37.5
	TAC	7	29.2	15	62.5
Lys	AAA	32	82.1	6	15.4
	AAG	7	17.9	33	84.6
Ile	ATT	20	43.5	6	13.0
	ATC	8	17.4	40	87.0
	ATA	18	39.1	0	0.0
***	TAA	1	100.0	1	100.0
	TAG	0	0.0	0	0.0
	TGA	0	0.0	0	0.0
Thr	ACT	11	37.9	0	0.0
	ACC	7	24.1	21	72.4
	ACA	9	31.0	8	27.6
	ACG	2	6.9	0	0.0
Pro	CCT	11	35.5	15	48.4
	CCC	3	9.7	4	12.9
	CCA	14	45.2	11	35.5
	CCG	3	9.7	1	3.2
Ala	GCT	13	37.1	3	8.6
	GCC	4	11.4	30	85.7
	GCA	14	40.0	0	0.0
	GCG	4	11.4	2	5.7
Gly	GGT	7	17.5	0	0.0
	GGC	9	22.5	34	85.0
	GGA	20	50.0	4	10.0
	GGG	4	10.0	2	5.0
Val	GTT	14	36.8	0	0.0
	GTC	6	15.8	5	13.2
	GTA	13	34.2	0	0.0
	GTG	5	13.2	33	86.8
Arg	AGA	8	40.0	8	40.0
	AGG	1	5.0	4	20.0
	CGT	6	30.0	0	0.0
	CGC	1	5.0	4	20.0
	CGA	3	15.0	0	0.0
	CGG	1	5.0	4	20.0
Ser	AGT	8	25.0	2	6.3
	AGC	7	21.9	13	40.6
	TCT	4	12.5	4	12.5
	TCC	1	3.1	13	40.6
	TCA	10	31.3	0	0.0
	TCG	2	6.3	0	0.0
Leu	CTT	13	25.0	1	1.9
	CTC	3	5.8	2	3.8
	CTA	9	17.3	1	1.9
	CTG	4	7.7	47	90.4
	TTA	15	28.8	0	0.0
	TTG	8	15.4	1	1.9

Table 6

Restriction enzyme site	Sequence before change	Sequence after change
35 BssSI	Ctggtg	Ctcgga
92 SsPI	Aatatt	Aatact
118 ClaI	Atcgat	Atcgac
146 NdeI	Catatg	Cctatg
155 SsPI	Aatatt	Gatttt
189 XbaI	Tctaga	Cctgga
282 EcoT14I	Ccaagg	Ccaggg
417 XbaI	Tctaga	Cctgga
460 EcoRV	Gatatc	Gacatc
524 ApoI	Aaattt	Aagttc
553 EcoT14I	Ccttgg	Ccctgg
570 PshBI	Attaat	Gctgat
769 AflIII	Cttaag	Ctgaag
790 ApoI	Aaattt	Aagttt
802 ApoI, EcoRI	Gaattc	Gagttc
955 EcoRV	Gatatc	Gacatc
1030 Aor51HI	Agcgct	Agcgcc
1076 MunI	Caattg	Ccatcg
1094 NdeI	Catatg	Cctatg
1117 EcoRV	Gatatc	Gacatc
1193 EcoRV	Gatatc	Gctacc
1217 BssSI	Ctcgt	Ccagg
1301 ClaI	Atcgat	Atcggc
1331 EcoRV	Gatatc	Gctacc
1381 SspI	Aatatt	Aacatc
1406 EcoRI, ApoI	Gaattc	Gcatcc
1410 AccIII	Tccgga	Cccaga
1417 ApoI	Gaattt	Gagttt
1605 SspI	Aatatt	Catctt
1613 SmaI	Cccggg	Cccgcg

Example 11

Vectors in which a wild-type or mutant luciferase gene was inserted downstream of three kinds of promoters (CMV, SV40 or CAG {CAG: (CMV enhancer)-(chicken β-action promoter)-(β-globin intron II)-(Kozak sequence)} were made (wild-type: CMV-Red, CAG-Red, mutant: CMV-REDm, CAG-REDm). At that time, the vectors in which an SKL sequence known as a peroxisome transfer sequence at the C-terminus had been deleted were made (wild-type: SV40-Red(-SKL), mutant: SV40-REDm(-SKL), CAG-REDm).

The NIH3T3 cells were transfected with each gene using Lipofectamine, and the luminescence activity in the cells after 24 hours was measured (Fig. 15). A luminescent substrate mixed solution (Toyo B-Net Co., Ltd.) and AB2500 supplied from ATTO Corporation were used as the substrate and as a luminescence determining apparatus, respectively for the measurement of the luminescence activity. A sample was made by adding 50 μL of PicaGene to 50 μL of a cell extract solution. The sample containing CMV-Red or SV40-Red(-SKL) showed a value of around 1000 RLU whereas the sample containing CMV-REDm or SV40-REDm(-SKL) showed a value of 2×10^7 to 4×10^7 RLU. As shown in Fig. 15, the high activity was observed in the sample containing CAG-Red, but in the sample containing its mutant, the activity was increased by about two times. The SKL sequence was believed to be involved in activity increase, but the activity in the sample containing SKL was increased by only several %. From these results, it has been demonstrated that CAG and REDm are useful as reporter genes for analysis of the expression of mammalian genes. By the technique in Example 10, it is possible to stably express the luciferase from rail road worm in the mammalian cells. Therefore, by the similar procedure, the sequence of the green-emitting luciferase gene from rail road worm was modified (SEQ ID NO:16). In the modified sequence, 16 transcription factor binding sites were modified in the wild-type, and it has 76% homology with the wild type.

Example 12

The luminescence spectrum of the red-emitting luciferase gene derived from the rail road worm, expressed in the mammalian cells was analyzed. To 15 μL of an extract solution of the cells (NIH3T3 derived from a mouse, Rat-1 derived from a rat, A543 cells from human) transfected with the CMV-REDm gene whose activity was the highest, 15 μL of PicaGene was added, and the luminescence spectrum was measured using a weak luminescence spectrum determining apparatus supplied from ATTO Corporation. As a reference, the luminescence spectrum in an extract solution of

silk worm insect cells transfected with the gene described in SEQ ID NO:3 was also measured. Fig. 16 shows the luminescence spectra expressed in the mouse NIH3T3 cells (bold line) and silk worm insect cells (thin line). The maximum luminescence wavelength in the mouse NIH3T3 cells was 630 nm and that in the silk worm insect cells was around 622 nm. These spectra were not affected by pH and the surrounding solution, and were always displayed as the same spectra. The maximum luminescence wavelength in Rat-1 cells from the rat and A543 cells from the human was also 630 nm.

Example 13

In order to stably express in the mammalian cells, in the sequences of wild-type *Rhagophthalmus ohba* green-emitting luciferase (the gene sequence and the amino acid sequence are shown in SEQ ID NOS:8 and 12, respectively.) and the wild-type *Rhagophthalmus ohba* orange-emitting luciferase (the gene sequence and the amino acid sequence are shown in SEQ ID NOS:9 and 13, respectively.), with keeping the followings in mind, constructs were artificially made.

1) Changes of 15 transcription factor binding sites (20 DNA sequences) (Table 7)

2) Changes of 322 DNA sequences for making the codon use frequency close to the mammalian codon usage (Table 8).

3) Changes of 30 common restriction enzyme sites (2 in 49 DNA sequence are the same as those of the transcription factor binding sites) (Table 9).

In Tables 8 and 9, RoLWT represents the wild-type *Rhagophthalmus ohba* luciferase, and RoLm represents the mutant *Rhagophthalmus ohba* luciferase.

The gene sequence of the resulting mutant *Rhagophthalmus ohba* green-emitting luciferase gene and the amino acid sequence thereof are shown in SEQ ID NOS:10 and 14, respectively. The gene sequence of the resulting mutant *Rhagophthalmus ohba* orange-emitting luciferase gene and the amino acid sequence thereof are shown in SEQ ID NOS:11 and 15, respectively.

The homology between the mutant *Rhagophthalmus ohba* green-

emitting luciferase gene sequence (SEQ ID NO:10) and the wild-type *Rhagophthalmus ohba* green-emitting luciferase gene sequence (SEQ ID NO:8) is 76.0% (Fig. 17)

Table 7

Predicted transcription factor	**Mutant number**	**Mutant sequence (Italic means a mutated part)**	
Activator protein 4	64 - 80	**(C69T)** **(G75C)**	cccagggacccccctggacctgggcaccgcc ggcat*t*cag ct*c*tacag agccctgaccaacttctccttcctgaggga
RAR-related orphan receptor alpha2	81 - 97	**(A81G)**	cctgggcaccgccggcatccagctgtacag *g*gccctgac caacttct ccttcctgagggaggccctgatcgacgccc
Nuclear factor 1	169 - 187	**(C183T)**	gtggtgtcttacgccgacatcctggagaac agctgtaga ctggc*t*aagt gctacgagaactacggcctgcgccagaac a
Progesterone receptor binding site	237 - 255	**(C243T)**	gcgccagaacagcgtgatctccgtgtgcag cgagaa*t*ag caccatcttc ttctaccccgtgatcgccgccctgtacat g
Tumor suppressor p53 (5' half site)	458 - 478	**(C462T)**	tcaagaaggtggtgctgctggacagcaagg agga*t*atgg gcgaggcccagt gcctgagcaacttcatggcccggtact ccg
Tumor suppressor p53 (5' half site)	563 - 583	**(G573T)** **(C576T)**	tcaagccaagggacttcgacgccaaggagc aggtggccc t*t*at*t*atgtcct cctctggcaccaccggcctgccaaagg gcg
Zinc finger transcription factor ZBP-89	850 - 864	**(C858T)** **(C861T)**	atcgagaagtacagaatcccaacaatcgtg ctggcccc*t* cc*t*gtg atggtgttcctggccaagagccccctggtg
Nuclear factor 1	865 - 883	**(C879T)**	atcccaacaatcgtgctggcccccccgtg atggtgttc ctggc*t*aaga gccccctggtggaccagtacgacctgtcc a
Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y	950 - 964	**(G960T)**	gagaggtggccaccggcggcgcccctgtgg gcaccgagg t*t*gccg tggccgtggccaagcggctgaagatcggcg
X-box-binding protein 1	1252 - 1266	**(C1263A)**	gccatcgacaaggagggctggctgcactcc ggcgacgtg gg*a*tac tacgacgacgatggccacttcttcgtggtg
H6 homeodomain HMX3/Nkx5.1 transcription factor	1278 - 1290	**(C1281A)** **(C1284T)**	ctccggcgacgtgggctactacgacgacga tgg*a*ca*t*tt cttc gtggtggaccggctgaaggagctgatcaag
Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)	1302 - 1322	**(G1308A)**	cgacgatggccacttcttcgtggtggaccg gctgaa*a*ga gctgatcaagta caagggctaccaggtggcccccgccga gct
Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	1385 - 1395	**(C1389T)**	Agtggctgctgctccagcacccatccatca agga*t*gccg gc gtgaccggcgtgcccgacgaggccgccggc
NF-kappaB (p50)	(1502-1516)	**(G1506A)** **(C1512T)**	ccgagcaggagatcatcgactacatcgccg agcg*a*gtgt c*t*ccca ccaagcgcatccggggcggcgtcgtcttcg
Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	1531 - 1541	**(C1536A)**	gagcgggtgtcccccaccaagcgcatccgg ggcgg*a*gtc gt cttcgtggacgacatccccaagggcgccac

5

Table 8

Amino Acid	Codon	RoLWT #	RoLWT %	RoLm #	RoLm %
Met	ATG	10	1.84	10	1.84
Trp	TGG	2	0.37	2	0.37
Glu	GAA	31	5.7	1	0.18
	GAG	5	0.92	35	6.43
Phe	TTT	11	2.02	0	0
	TTC	13	2.39	24	4.41
Asp	GAT	15	2.76	3	0.55
	GAC	12	2.21	24	4.41
Cys	TGT	5	0.92	1	0.18
	TGC	5	0.92	9	1.65
His	CAT	8	1.47	1	0.18
	CAC	2	0.37	9	1.65
Gln	CAA	9	1.65	0	0
	CAG	4	0.74	13	2.39
Asn	AAT	11	2.02	1	0.18
	AAC	7	1.29	17	3.12
Tyr	TAT	11	2.02	0	0
	TAC	8	1.47	19	3.49
Lys	AAA	28	5.15	1	0.18
	AAG	12	2.21	39	7.17
Ile	ATT	19	3.49	2	0.37
	ATC	10	1.84	34	6.25
	ATA	7	1.29	0	0
***	TAA	1	0.18	1	0.18
	TAG	0	0	0	0
	TGA	0	0	0	0
Thr	ACT	9	1.65	0	0
	ACC	13	2.39	30	5.51
	ACA	5	0.92	2	0.37
	ACG	5	0.92	0	0
Pro	CCT	6	1.1	4	0.74
	CCC	8	1.47	16	2.94
	CCA	7	1.29	4	0.74
	CCG	4	0.74	0	0
Ala	GCT	14	2.57	4	0.74
	GCC	10	1.84	35	6.43
	GCA	8	1.47	0	0
	GCG	6	1.1	0	0
Gly	GGT	6	1.1	0	0
	GGC	7	1.29	34	6.25
	GGA	18	3.31	5	0.92
	GGG	8	1.47	0	0
Val	GTT	14	2.57	1	0.18
	GTC	10	1.84	3	0.55
	GTA	17	3.12	1	0.18
	GTG	6	1.1	42	7.72
Arg	AGA	8	1.47	9	1.65
	AGG	4	0.74	7	1.29
	CGT	3	0.55	0	0
	CGC	4	0.74	3	0.55
	CGA	5	0.92	1	0.18
	CGG	2	0.37	6	1.1
Ser	AGT	6	1.1	0	0
	AGC	8	1.47	14	2.57
	TCT	7	1.29	3	0.55
	TCC	3	0.55	17	3.12
	TCA	3	0.55	0	0
	TCG	7	1.29	0	0
Leu	TTA	19	3.49	0	0
	TTG	16	2.94	0	0
	CTT	12	2.21	1	0.18
	CTC	1	0.18	4	0.74
	CTA	3	0.55	0	0
	CTG	6	1.1	52	9.56

Table 9

Restriction enzyme site		RoLWT	ROLm
35	AvaI	CTCGAG	CCAGGG
35	XhoI	CTCGAG	CCAGGG
59	PstI	CTGCAG	CCGCCG
65	ApoI	GAATTC	GCATTC
65	EcoRI	GAATTC	GCATTC
70	MunI	CAATTG	CAGCTC
90	ApoI	GAATTT	CAACTT
438	ScaI	AGTACT	GGTGCT
528	ApoI	AAATTT	AAACTT
532	DraI	TTTAAA	TTCAAG
618	HincII	GTTAAC	GCTGAC
618	HpaI	GTTAAC	GCTGAC
630	ApoI	AAATTT	GAACCT
660	BamHI	GGATCC	GGACCC
744	Psp1406I	AACGTT	AACCCT
793	BspT104I	TTCGAA	TTCGAG
810	AflII	CTTAAG	CCTGAG
833	ApoI	GAATTC	GAATCC
833	EcoRI	GAATTC	GAATCC
931	AgeI	ACCGGT	ACCGGC
1038	PshBI	ATTAAT	GCTGAT
1050	BspHI	TCATGA	CCACGA
1113	BglII	AGATCT	GGACCT
1165	DraI	TTTAAA	TTCAAG
1225	ClaI	ATCGAT	ATCGAC
1273	PshAI	GACGATGGTC	GACGATGGAC
1296	PvuI	CGATCG	GGACCG
1302	DraI	TTTAAA	GCTGAA
1328	EcoRV	GATATC	GCTACC
1523	Bst1107I	GTATAC	GCATCC

Example 14

Vectors in which the wild-type or mutant luciferase gene (orange- or green-emitting from *Rhagophthalmus ohba*) was inserted downstream of three kinds of the promoters SV40 were made (wild-type: SV40-RoL (Green) and SV40-RoL (orange), mutant: SV40-RoL (Green)m and SV40-RoL (Orange)m). The cultured fibroblast cells, NIH3T3 cells were transfected with each gene using Lipofectamine

Plus, and the luminescence activity in the cells after 24 hours was measured. A luminescent substrate mixed solution (supplied from Toyo B-Net Co., Ltd.) and LB9506 supplied from Berthold were used as a substrate and as a luminescence determining apparatus, respectively. A sample was made by adding 50 μL of PicaGene to 50 μL of a cell extract solution. As a result, the samples containing the wild type SV40-RoL (Green) and SV40-RoL (orange) exhibited values of about 1×10^6 and 4×10^5 RLU, respectively whereas the samples containing mutant SV40-RoL (Green)m and SV40-RoL (Orange)m exhibited values of 5×10^8 and 8×10^7 RLU, respectively. Comparing the wild type with the mutant, when the value of the wild-type is made 1, the activity values were increased by about 44 times and about 57 times in the mutant green and orange luciferases, respectively. These results demonstrate that the mutant is useful as the reporter gene for the analysis of the mammalian gene expression.

Example 15

An outline of a method for simultaneously determining the transcription activities of three genes in the mammalian cells using one substrate is shown in Fig. 19. Three gene vectors in which the promoter sequence has been inserted upstream of the red-emitting luciferase gene from the rail road worm, the green-emitting luciferase gene from *Rhagophthalmus ohba* and the orange-emitting luciferase gene from *Rhagophthalmus ohba* are co-expressed in the cultured cells. The co-expressing cells after a certain time course after the treatment of the cells are lysed. Subsequently, three transcription activities are measured by separating the luminescence activities of red-emitting luciferase from the rail road worm, the green-emitting luciferase from *Rhagophthalmus ohba* and the orange-emitting luciferase from *Rhagophthalmus ohba* in the cell extract solution using the color filters. Thus, 15 μL of PicaGene was added to 15 μL of the extract solution of the cells in which the red-emitting luciferase gene from the rail road worm, the green-emitting luciferase gene from *Rhagophthalmus ohba* and the orange-emitting

luciferase gene from *Rhagophthalmus ohba* were independently expressed. Then the luminescence spectra were measured using the weak luminescence spectrum determining apparatus supplied from ATTO Corporation (Fig. 20). As a result of examining the luminescence spectra, it has been confirmed that color split is possible by selecting the color filter O-54 type supplied from Hoya Co., Ltd. for splitting the green and orange lights and selecting the color filter R-60 type supplied from Hoya Co., Ltd. for splitting the orange and red lights. The measuring procedure is as follows. (1) The emitted light intensity is measured without use of the filter (luminescence activities of the red, orange and green-emitting luciferases). (2) The color filter O-54 type is inserted in a luminometer and the emitted light intensity is measured to yield the luminescent activity of the green-emitting luciferase. (3) The color filter R-60 type is inserted in the luminometer and the emitted light intensity is measured to yield the luminescent activity of the green and orange luminescence activity. (4) The luminescence activity of the red is calculated by converting the transmittance of the filters. Further, the activities of three color luciferases are corrected. This way, it is possible to evaluate the emitted lights intensities and the abundance of the red-, green- and orange-emitting luciferases.

Example 16

It has been examined in the model experiment whether two enzymes with different compositions in the red-, orange- and green-emitting luciferases whose abundance ratios are different can be quantified by the procedure determined in Example 15. In Fig. 21 for the red- and green-emitting luciferases (A), the green- and orange-emitting luciferases (B), and the orange- and red-emitting luciferases (C), the luminescence activities in samples with different abundance ratios were obtained by (1) measuring all emitted light intensities, (2) using the set filter and converting. As a result, it has been demonstrated that the luminescence activity is changed in a linear relationship with

the abundance ratio. This suggests that the different amounts of the red-, orange- and green-emitting luciferases expressed in the mammalian cells can be quantified by the luminometer in which the filter has been inserted.

Example 17

It has been examined in the model experiment whether two enzymes with different compositions can be quantified by making the amount of one light-emitting enzyme constant in the red-, orange- and green-emitting luciferases whose abundance ratios are different by the procedure determined in Example 15. In Fig. 22, for the orange- and green-emitting luciferases by making the red-emitting luciferase constant (A), for the green- and red-emitting luciferases by making the orange-emitting luciferase constant (B), and for the orange- and red-emitting luciferases by making the green-emitting luciferase constant (C), the luminescence activities in samples with different abundance ratios were obtained by (1) measuring all emitted light intensities, (2) using the set filter and converting. As a result, it has been demonstrated that the luminescence activity is changed in a linear relationship with the abundance ratio. This suggests that the different amounts of the red-, orange- and green-emitting luciferases expressed in the mammalian cells can be quantified by the luminometer in which the filter has been inserted. Therefore, it has been demonstrated that it is possible to quantify the three luciferases by one substrate, and that the amounts of transcription activities of three genes can be measured.